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PRINCIPAL INVESTIGATOR: **Robert A. Weinberg, Ph.D.**

CONTRACTING ORGANIZATION: **Whitehead Institute for Biomedical Research, Cambridge, MA 02142**

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**Generation of Breast Cancer Stem Cells by the EMT
Robert A. Weinberg, Ph.D.**

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Introduction

The progression of benign human breast cancer cells into highly malignant derivatives is accompanied by the acquisition of mesenchymal cell characteristics, motility, and invasiveness. These multifaceted changes in cell phenotypes are involved in the transformation program termed the epithelial-mesenchymal transition (EMT). Recently, we discovered that in addition to conferring cell-biological traits of high-grade malignancy, the EMT program also converts more differentiated mammary epithelial cells into derivatives having many of the attributes of epithelial stem cells, a conversion that operates in both immortalized and transformed, tumorigenic mammary epithelial cells. However, it remained unclear whether. The outcome of these experiments holds important implications for the mechanisms controlling the formation of normal mammary epithelial stem cells as well as the formation of breast cancer stem cells. In this study period, we have gained substantial insight into the identities of the master regulators of mammary stem cells.

Body

Our previous work demonstrated that differentiated mammary epithelial cells (MECs) that have undergone an epithelial-mesenchymal transition (EMT) acquire many properties of mammary stem cells, including the expression of stem-cell-surface markers and the ability to form mammospheres. In this study, we posed the questions of whether forced passage through an EMT can indeed form normal mammary gland stem cells (SCs), which can reconstitute an entire mammary ductal tree upon being transplanted into a cleared mammary fat pad, and whether the EMT-inducing transcription factors can act as master regulators of mammary SCs.

In the previous progress report cycle, we found that mammary stem cells specifically express the EMT-inducing Slug transcription factor (TF) relative to luminal progenitor cells or fully differentiated luminal cells (~90 fold higher in mammary SCs). This suggested to us that Slug was likely to be an excellent candidate for the regulators of the mammary SC state. We therefore asked whether induction of an EMT by Slug in mammary epithelial cells could generate mammary SCs. To test this, we transiently expressed Slug in MECs for 10 - 12 days using tetracycline-inducible lentiviral constructs, and subjected the cells to the *in vivo* gland-reconstitution assay. We found that cells having undergone an EMT induced by Slug had 40 – to 100-fold higher mammary gland-reconstituting activity. This experiment showed that induction of an EMT indeed leads to generation of bona fide mammary stem cells. However, because this experiment was done with MECs containing pre-existing SCs, we could not rule out the possibility that Slug may expand pre-existing SCs, rather than converting non-stem cells into stem cells.

In the past year or so, we focused on convincingly demonstrating that the EMT is able to convert non-stem cells into mammary SCs. First, we isolated either luminal progenitor cells or fully differentiated luminal cells that are devoid of mammary stem cells by FACS on the basis of CD49f and CD61. We then tested whether Slug could generate SCs in each non-stem cell population. We infected the cells with the tetracycline-inducible Slug construct or control vector and induced Slug expression for 5 days in adherent culture, and then stopped the doxycycline treatment and subjected the cells to an *in vitro* 3D Matrigel culture system in order

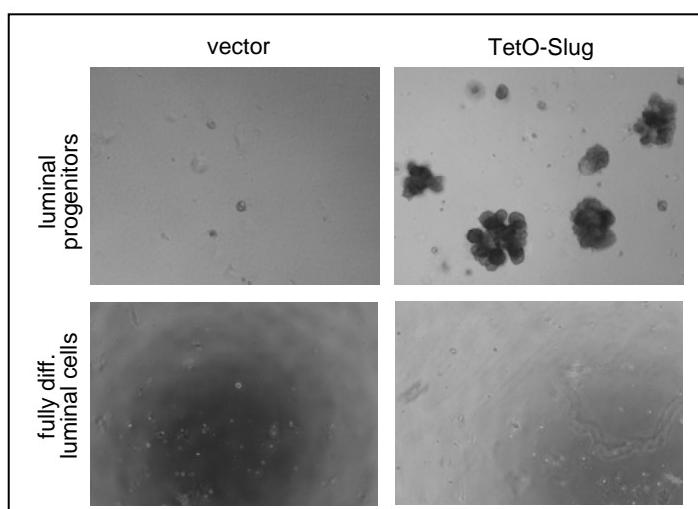


Figure 1: Luminal progenitor cells (top) or fully differentiated luminal cells (bottom) were infected with control or Slug vectors. After a 5-day treatment of doxycycline, cells were subjected to 3D matrigel culture to assay for mammary stem-cell activity.

to assay mammary SC activity. In this type of assay, mammary stem cells can form complex 3D ductal-lobular organoids. As shown in Figure 1, transient expression of Slug efficiently induced mammary stem cells from luminal progenitor cells, however it failed to induce mammary stem cells from fully differentiated luminal cells. This experiment demonstrated that induction of an EMT by Slug can indeed generate stem cells from non-stem cells; however it could only do so in the progenitor cells. We reasoned that relative to luminal progenitor cells, the fully differentiated luminal cells may lack certain factor(s) that can cooperate with Slug to induce mammary SC state.

We therefore gathered a dozen or so TFs that have been associated with stem cell biology in general or factors that have been shown to act together with EMT-inducing TFs during developmental processes. We co-expressed each individual factor with Slug in fully differentiated luminal cells in order to screen for factors that can act together with Slug to induce stem-cell activities in the fully differentiated cells. We found that co-expression of Sox9 with Slug can robustly induce stem-cell activity in the fully differentiated cells (Figure 2), while expression of either Slug or Sox9 alone failed to induce mammary SC. We analyzed the expression pattern of Sox9 in different cell types of mammary epithelium using a strain of Sox9-GFP reporter mice. We found that Sox9 is highly expressed in the luminal progenitor cells and a subset of basal cell population that contains stem cells. The fact that only luminal progenitors but not fully differentiated luminal cells express Sox9 explains why Slug expression can generate mammary SCs only in luminal progenitor cells but not in fully differentiated cells.

We further asked whether Sox9 and Slug indeed convert fully differentiated luminal cells to mammary SCs that can regenerate an entire mammary ductal tree. We infected the fully differentiated luminal cells with tetracycline-inducible Slug and Sox9 and induced the expression of constructs by doxycycline for 5 days in adherent culture, and then transplanted the cells in a 1:1 mixture with RFP-expressing control MECs in cleared mammary fat pads. As anticipated, the control vector-expressing luminal cells had no reconstitution activity at all. In stark contrast, the

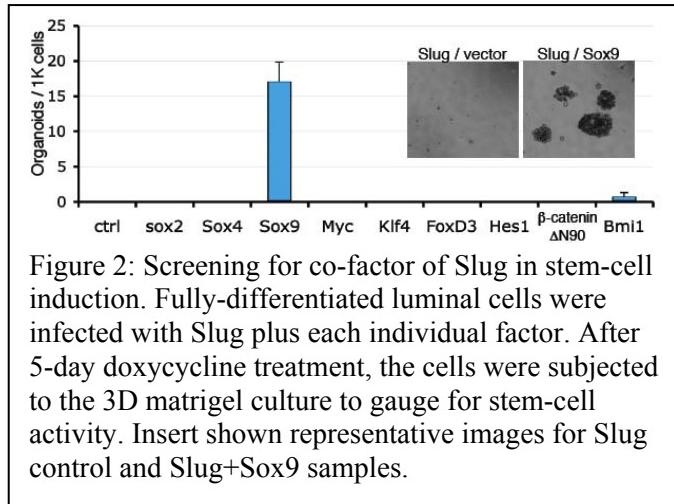


Figure 2: Screening for co-factor of Slug in stem-cell induction. Fully-differentiated luminal cells were infected with Slug plus each individual factor. After 5-day doxycycline treatment, the cells were subjected to the 3D matrigel culture to gauge for stem-cell activity. Insert shown representative images for Slug control and Slug+Sox9 samples.

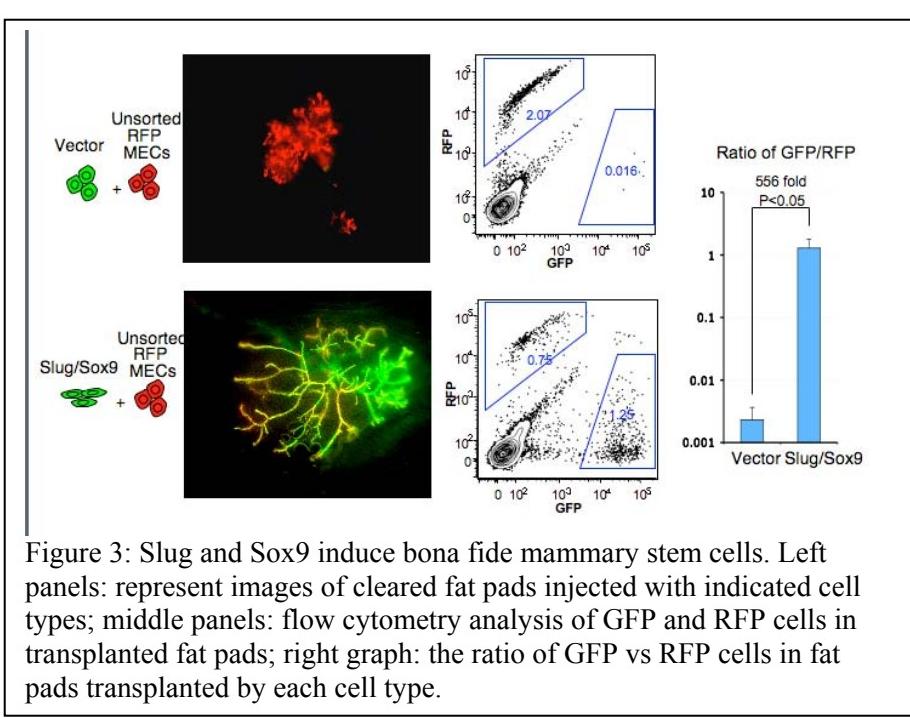


Figure 3: Slug and Sox9 induce bona fide mammary stem cells. Left panels: represent images of cleared fat pads injected with indicated cell types; middle panels: flow cytometry analysis of GFP and RFP cells in transplanted fat pads; right graph: the ratio of GFP vs RFP cells in fat pads transplanted by each cell type.

Slug/Sox9-expressing luminal cells reconstituted the fat pads robustly (Figure 3).

The above experiments demonstrated that the EMT in collaboration with Sox9 can convert even the fully differentiated MECs – cells at the bottom of differentiation hierarchy – back into a bona fide stem-cell state. We further asked whether Slug and Sox9 are required for maintaining the pre-existing stem cells in the mammary epithelial cell population. We knocked down the expression of Slug or Sox9 in unfractionated MECs using lentivirus vector-expressed shRNAs and then gauged mammary stem-cell activity of these cells using both in vitro or in vivo assays. In both assays, knocking down of either genes leads to severe to complete inhibition of mammary stem-cell activity (13-fold decrease to complete inhibition). These data demonstrated that EMT-inducing factor Slug and Sox9 act as master regulators of mammary stem-cell state. They are both sufficient in inducing mammary SCs from non-SCs and necessary for maintaining pre-existing SCs.

Key Research Accomplishments

- The induction of an EMT has now been demonstrated to convert a normal murine mammary epithelial cells into mammary epithelial stem cells that can, following orthotopic implantation, generate an entire mammary ductal tree. This observation offers us for the first time a definitive proof of the connection between EMT and entrance into a stem-cell state. The EMT-inducing transcription factor Slug acts as master regulator for the stem-cell state.
- Genetic screens have identified Sox9 as an important co-factor for Slug-mediated stem-cell induction and maintenance.

Reportable Outcomes

This supported the following publications:

- Valastyan, S., Reinhardt, F., Benaich, N., Calogrias, D., Sza'ad, A.M., Wang, Z.C., Brock, J.E., Richardson, A.L., and Weinberg, R.A. (2009) A pleiotropically acting MiRNA, miR-31, inhibits breast cancer metastasis. *Cell*, 137: 1032-1046. PMCID: PMC2766609
- Valastyan, S., Benaich, N., Chang, A., Reinhardt, F., and Weinberg, R.A. (2009) Concomitant repression of three target genes can explain the impact of a microRNA on metastasis. *Genes and Development*, 23:1-6 PMCID: PMC2779763
- Valastyan S, Chang A, Benaich N, Reinhardt F, Weinberg RA.(2010) Concurrent suppression of integrin alpha5, radixin, and RhoA phenocopies the effects of miR-31 on metastasis. *Cancer Res*. 2010 Jun 15;70(12):5147-54. Epub 2010 Jun 8. PMCID: PMC2891350

Conclusion

One major implication of the reported work derives from experiments reported herein in which we have succeeded in greatly increasing the proportion of normal mammary epithelial stem cells in a population of differentiated mammary epithelial cells, doing so by expressing EMT-inducing transcription factors in these cells transiently. Since the EMT can otherwise be induced by contextual signals that have been and are being identified, this suggests that a protocol can be developed involving exposure of differentiated epithelial cells to a mixture of EMT-inducing signals that can substantially increase the proportion of normal epithelial stem cells in these cultures. This holds, in turn, important implications for future modalities of regenerative therapy designed to restore damaged epithelial tissues through implantation of normal, syngeneic epithelial stem cells.

A second major outcome of this work is that we have now started to understand the core transcription regulatory network of mammary stem cells by identifying the master regulators of the

Weinberg, Robert A. (BC073843)

mammary stem-cell state. This core stem-cell transcription network may also play important role in determining the cancer stem cell state, which is being studied intensively by us now.